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SHORT COMMUNICATION

Assessment of genetic diversity in *Dalbergia sissoo* clones through RAPD profiling

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Abstract: We studied the genetic polymorphism among 29 clones of shisham (*Dalbergia sissoo* Roxb) belonging to different geographic regions using random amplified polymorphic DNA (RAPD) markers. Out of 30 primers used, only 20 primers generated polymorphism in amplified product. In total 232 bands were amplified with 20 primers, of which 192 (82%) were polymorphic with an average of 9.6 bands/primer. The resolving power (Rp) ranged from 2.14 (Primer 5) to 11.93 (Primer 4). Primer 4 and Primer 3 possessed high Rp value. Polymorphism information content (PIC) ranged from 0.15 (Primer 5) to 0.37 (Primer 4). Primer 4 amplified total 18 bands in 29 genotypes with PIC value of 0.37 hence; this set of primer was most informative. The similarity coefficient analysis revealed two clusters. The first cluster comprised of only 10 clones and the second major cluster comprised of 19 clones. The genetic similarity among 29 clones ranged from 25.86% (clone 10 and 235) to 100% (clone 19 and 59), suggesting a wide genetic base in shisham clones.

Keywords: clones; clonal seed orchard; *Dalbergia sissoo*; RAPD; polymorphism information content; resolving power

Introduction

Dalbergia sissoo Roxb. is the best known timber species of Rose wood genus (Family Leguminoceae, sub family Fabaceae). It is one of the seven most valuable timber species in Asia and indigenous to India and Burma. *D. sissoo* is native to the Himalayan foothills in India, Pakistan and Nepal. This species is widely distributed in many parts of India at elevations of 900–1 500 m in sub-Himalayan tracts. It grows naturally in new alluvial deposits but prefers well drained sandy loam soils with adequate moisture supply.

D. sissoo with crooked trunk and light crown is the most favoured species for plantations due to its fast growing rate and various uses. For example, the species enriches soil through atmospheric nitrogen fixation by Rhizobium bacteria in nodule rich roots and with fast decomposing leaves. Unfortunately, the population of this species is declining fast due to heavy mortality.

In a species like sissoo where wide diversity has not yet been touched, quantitative assessment of genetic diversity present among selective genotypes would be useful for future breeding and tree improvement programmes. Traditionally, the assessment of the genetic composition of germplasm is conducted on the basis of morphological, biochemical, phenological and physiological characters that are blurred by environmental influences. In the last decade, Mendelian genetic markers have been used for many types of genetic analysis in forest genetics and tree improvement research at DNA level, allowing an estimation of the degree of relatedness between individuals. Among various molecular markers, RAPD markers have been extensively used for detecting genetic variation, comparative evaluation of genetic relationships among the clones and species, identification and classification of forest trees (Gautam 2006, 2007; Arif et al. 2009; Singh et al. 2009). RAPD-PCR analysis is potentially simple, rapid, and reliable, and this method requires small amount of DNA for analysis. Present study reports the extent of diversity among different genotypes of *D. sissoo* growing in a clonal seed orchard (CSO) at Lacchiwala through RAPD profiling.

Materials and method

Plant material

Under World Bank FREE project, a clonal seed orchard (CSO) of *D. sissoo* was established in year 1996 at Lacchiwala, Dehradun, India. The geographical details and soil characters of the site are described in Table 1. The experimental material comprised of 31 genotypes of *D. sissoo* tree collected from diverse

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locations of India and Nepal. The geographical distributions of collection sites are represented in Table 2. The plantation trials were laid out in Randomised Block Design with three replications. Each replicate consisted of nine ramets at a spacing of 6m× 6 m.

Table 1. Site characteristics of clonal seed orchard (Lacchiwala)

Geographical parameter		Soil parameter	
Latitude	30°1″60N	pH	8
Longitude	78°07″0E	E.C. (ms)	0.26
Altitude	512 m	Organic Carbon (%)	0.27
		Nitrogen Con. (Kg/ha.)	137.9
		Potassium Con.(Kg/ha.)	22.40
		Phosphorus (Kg/ha)	48.4
		Calcium con. (ppm)	170.0
		Magnesium (ppm)	122.0

Table 2. Geographical details of *Dalbergia sissoo* clones at Lacchiwala

S.No.	Clone No	State	Circle	Division	Range
1	6	Uttarpradesh	Bijnor	Chiriyapur	Sabalgarh
2	10	Uttarpradesh	Haridwar	Pathri	
3	18	Uttarpradesh	Saharanpur	Shah Mansoorpur (Saharanpur)	Saharanpur
4	19	Uttarpradesh	Saharanpur	Khanpur	Saharanpur
5	42	Uttarpradesh	Gonda	Tulsipur	Trilokpur
6	59	Haryana	-	Ambala	Khalawala
7	60	Haryana	-	Ambala	Khalawala
8	66	Haryana	-	Yamuna Nagar	Chichrauli
9	67	Haryana	-	Yamuna Nagar	Chichrauli
10	78	Rajasthan	-	Sriganga Nagar	Hanumangarh
11	80	Rajasthan	-	Sriganga Nagar	Hanumangarh
12	84	Rajasthan	-	Sriganga Nagar	Hanumangarh
13	85	Rajasthan	-	Sriganga Nagar	Hanumangarh
14	87	Rajasthan	-	Shregarh	Hanumangarh
15	88	Rajasthan	-	Shregarh	Hanumangarh
16	89	Rajasthan	-	Shregarh	Hanumangarh
17	92	Rajasthan	-	Lakhanwali village	Hanumangarh
18	93	Rajasthan	-	Lakhanwali village	Hanumangarh
19	94	Rajasthan	-	Lakhanwali village	Hanumangarh
20	103	Rajasthan	-	Hanumangarh	Suratgarh
21	123	Nepal	-	Sagarnath	Sagarnath
22	189	Uttarpradesh	Gonda	Gonda	Janakpur
23	192	Uttarpradesh	Gonda	Gonda	Hasnapur
24	193	Uttarpradesh	Gonda	Gonda	Hasnapur
25	194	Uttarpradesh	Gonda	Gonda	Hasnapur
26	196	Uttarpradesh	Gonda	Gonda	Hasnapur
27	198	Uttarpradesh	Gonda	Gonda	Hasnapur
28	202	Uttarpradesh	Gonda	Gonda	Hasnapur
29	235	Uttarpradesh	Gonda	Gonda	Banketwa

Genomic DNA extraction

Fresh leaves were collected from individual clone and brought to Plant Physiology laboratory in ice boxes. They were stored at -20°C in freezer (Vest Frost DFS 345). The genomic DNA was extracted with CTAB (Cetyltrimethyl ammonium bromide) method (Stange et al. 1998). Extracted DNA was quantified on biophotometer (Eppendorf AG 22331 Hamburg).

DNA amplification

All samples were brought down to a uniform concentration of 5ng/µl to be used as template DNA for PCR cock tail. Genomic DNA was amplified through PCR using RAPD primers in Techne Thermocycler (FPR OG 05 D): initial denaturation at 94°C for 5 min, followed by 44 cycles of denaturation at 94°C for 1 min, primer annealing for 1 min at respective temperature, extension at 72°C for 2 min, and termination by 5 min at 72°C. Initially 30 random decamer primers were used for amplification of a set of 10 diverse genotypes. Twenty primers showing amplification in the representative set were later used for amplification of whole set of genotypes. PCR amplification was performed in a 25-µl reaction volume, containing 16.67 µl Millipore water, 2 µl of 10X Taq buffer B (Genei, METB2), 2 µl of 10X $MgCl_2$ (Genei, METD2), 2 µl of 10 mM dNTPs mix 2.5 mM each (Fermentas), 1 µl of 20 mM primer, 0.33 µl of 3 units/ µl Taq DNA polymerase (Fermentas), and 1 µl of 5 ng/µl of genomic DNA working solution. Subsequently amplified products were electrophoresed using 1.5% agarose gel (Genei, Low EEO) with 1x TBE buffer at pH 8.0 for 3 h using horizontal electrophoresis system (Genei). Gels were visualized by Ethidium bromide staining and photographed under UV light using Gel Documentation System (I.T. System UVP, Digi Doc). Details and nucleotide sequence of RAPD primers (Synthesized by Operon Biotechnologies) are given in Table 3.

Table 3. RAPD primers and their sequences used for study

S. No.	Primer sequence (5' – 3')	GC Content (%)
1	GTGAGGCGTC	70
2	ACTCAGCCAC	60
3	GGGGGTTAGG	70
4	GTGTGCCCCA	70
5	CAGGCCCTTC	70
6	TGC CGA GCTG	70
7	AAT CGG GCTG	60
8	TCT GTG CTGG	60
9	AGC CAG CGAA	60
10	GTT GCG ATCC	60
11	AGG GGT CTT G	60
12	GGT CCC TGA C	70
13	GAA ACG GGT G	60
14	GTG ACG TAG G	60
15	GGG TAA CGC C	70
16	GTG ATC GCA G	60
17	CAA TCG CCG T	60
18	GAC CGC TTG T	60
19	AGG TGA CCG T	60
20	TGC CGA GCTG	70

The RAPD allele sizes were determined based on bands relative to the ladder (φX 174 DNA Hac III Digest, 1 KB DNA ladder, Genei). Each primer of RAPD reaction was repeated thrice to ensure reproducibility and reliability of RAPD markers. Only reproducible bands in each of three replications were considered.

Data analysis

The amplified bands in the whole germplasm set was recorded in a binary quantitative matrix as 1 (band present) and 0 (band absent). Reproducible amplified fragments of RAPD (bands present in each repetitions of each sample) were scored. Weak bands of negligible intensity and smeared bands were excluded from final data analysis. The ability of primer to distinguish between large number of genotypes i.e. resolving power of the primer (Rp) of RAPD primers were determined as described by Prevost (1999). Polymorphic information content (PIC) expresses the discriminatory power of the locus taking into account number of alleles expressed, their relative frequency and frequency of allele/ locus. The PIC values for each of 20 primers were estimated using the formula of Lynch. (1998).

$$PIC = \sum_{j=1}^{n} Pij^2$$

where Pij is the frequency of j^{th} allele in the i^{th} primer.

Cluster analysis

The RAPD primer amplification profiles (Fig. 1) of 29 clones were used to estimate genetic diversity/ relatedness based on number of shared amplified bands. All the amplified bands scored as present or absent were used as an index of genetic diversity/ relatedness. Pair wise comparisons of genotypes were employed to calculate Jaccard's similarity coefficient (Jaccard 1908). A dendrogram was constructed using unweighted pair group method with arithmetic average (UPGMA) using SHAN module of software NTSYS version (Rohlf 1998).

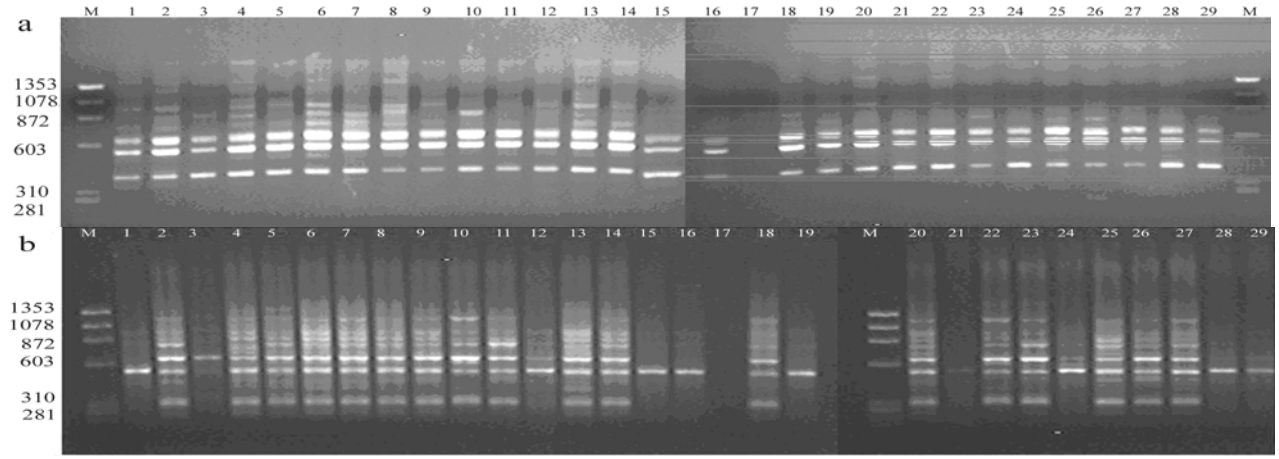

Fig. 1 RAPD amplification profile of 29 clones of *Dalbergia sissoo* with primer 1 and primer 5. M = marker φX 174 DNA Hac III Digest; Lane 1 – 29 are *Dalbergia sisoo* clones (06 to 235 in increasing order as like as arranged in Table 2.)

Results and discussion

RAPD analysis was conducted using single 10-base pair oligonucleotides primer reaction. Of the 30 primers screened, 20 primers (Operon technologies) amplified DNA. Primers used for genotyping the clones and their base sequence and GC content are listed in Table 3. The amplification product ranged from 0.28 to 0.138 Kb. No amplification was achieved in clone 92 hence only 29 genotypes were considered. The numbers of bands amplified ranged from 7 to a maximum of 18. In total 232 bands were amplified with 20 primers, with an average of 11.6 bands/ primer. Out of 232 bands amplified, 192 (82%) were polymorphic with an average of 9.6 bands/ primer and 40 (18%) were monomorphic with an average of 2 bands/ primer. As potential polymorphic markers, the highest number of polymorphic bands was obtained with Primer 2 and Primer 3 and the lowest with Primer 5. Two RAPD primers Pr-2 and Pr-3 showed 100% polymorphism, whereas, Pr-5 was least polymorphic (45%). The resolving power (Rp) of the 20 RAPD primers ranged from 2.14 (Pr-5) to 11.93 (Pr-4) with an average of 5.84 per primer. RAPD primer viz. Pr- 4 and Pr-3 possessed high Rp value of 11.93 and 6.48, respectively (Table 4). The primers with the high Rp values were more informative as they were able to distinguish more number of genotype. Polymorphism information content ranged from 0.15 (Pr-5) to 0.37 (Pr-4) with an average of 0.30 per primer. Pr-4 amplified total 18 bands in 29 genotypes with PIC value of 0.37; hence, this set of primer was most informative.

Cluster analysis

The genetic relatedness among 29 clones is presented in the form of a dendrogram (Fig. 2) based on data analysis of RAPD profile.

At 55% similarity level, the dendrogram revealed two clusters. The first cluster is represented by 10 clones (6, 123, 193, 94, 18, 84, 88, 89, 202 and 235) at over all similarity coefficients of 65%. The genotypes C6 and C123 were clustered into a sub group with 87.9% similarity coefficient. Group II is further sub clustered in three sub groups named as 2a, 2b and 2c. Sub cluster 2a comprised of clone 10 and 103 with 89.7% similarity coefficient. 2b sub cluster consisted of sub groups clone 59 and 42, 60 and 67, 189 and 192 at similarity coefficient of 94.8, 93.1 and 79.3 respectively, but C19 and C59 were similar at similarity coefficient of 100% belonging to Saharanpur & Ambala regions which are close to each other. Rest of the clones C87, 85, 93, 78, 66 and 80 formed individual cluster under 2b sub to sub cluster. Clones 194, 196 and 198 constituted one sub group 2c. Clone 196 and 198 belonging to Gonda were similar at similarity coefficient of 88%. The major group included the genotypes both from distantly and nearly located geographical regions, indicating thus that geographical location may not be the true index of genetic diversity. Considerable similarity coefficient range was observed among Shisham clones, ranging from 25.86% to 100%. A coefficient range of 0.563−0.946 was discernible in the results of Arif et al. (2009) in *D. sissoo*. Such a wide range in similarity coefficient values suggests that *D. sissoo* germplasm is genetically diverse. Similar observations were made by Gautam (2006, 2007).

Table 4. RAPD primers with sequences and the properties of amplified products of Shisham clones

S.No.	Primer	Primer sequence (5' – 3')	Total number of bands	Number of polymorphic bands	Polymorphism percentage	Resolving power	Polymorphic content	information
1	1	GTGAGGCGTC	10	7	70	4.93	0.31	
2	2	ACTCAGCCAC	7	7	100	3.74	0.36	
3	3	GGGGGTTAGG	12	12	100	6.48	0.36	
4	4	GTGTGCCCCA	18	17	94.44	11.93	0.37	
5	5	CAGGCCCTTC	11	5	45.45	2.14	0.15	
6	6	TGC CGA GCTG	8	6	75	5.32	0.40	
7	7	AAT CGG GCTG	12	10	83.33	6.22	0.30	
8	8	TCT GTG CTGG	9	7	77.78	6.10	0.30	
9	9	AGC CAG CGAA	14	12	85.71	5.70	0.35	
10	10	GTT GCG ATCC	15	13	86.67	5.90	0.20	
11	11	AGG GGT CTT G	15	13	86.67	5.30	0.35	
12	12	GGT CCC TGA C	13	11	84.62	5.90	0.30	
13	13	GAA ACG GGT G	9	7	77.78	5.20	0.20	
14	14	GTG ACG TAG G	10	9	90	5.75	0.20	
15	15	GGG TAA CGC C	11	8	72.73	6.12	0.30	
16	16	GTG ATC GCA G	14	12	85.71	5.50	0.35	
17	17	CAA TCG CCG T	12	10	83.33	6.10	0.30	
18	18	GAC CGC TTG T	10	8	80	6.40	0.25	
19	19	AGG TGA CCG T	9	8	88.89	6.10	0.30	
20	20	TGC CGA GCTG	13	10	76.92	6.20	0.30	
Total			232	192			-	
Average			11.6	9.6	82.25	5.85	0.30	

The groupings of the genotypes did not correspond to their eco-geographical regions. For example, genotypes of Pathri, Saharanpur, Nepal, Rajasthan and Gonda remained together. The present study revealed that the phenotypically similar genotypes could be reliably distinguished genetically using RAPD markers. The study thus displayed wide genetic base in *D. sissoo* clones. The dendrogram from RAPD profile recorded by unweighted pair group method with arithmetic average (UPGMA) cluster analysis could readily separate 29 genotypes of *D. sissoo* into different groups. It is advocated that DNA markers such as RAPDs could be used effectively for genetic diversity analysis in *D. sissoo*. RAPD profile has been used often for developing phylogenetic relationship (Rout 2003; Nanda 2004; Gautam 2007; Arif et al. 2009).

The use of RAPD marker for genetic variation studies is well documented (Chandra A. 2007; Vierling 1992). Gautam (2006) discriminated 30 clones of *D. sissoo* by RAPD analysis using decamer RAPD primers. Arif et al. (2009) compared 30 polymorphic primers (15 ISSR and 15 random) to study genetic diversity of 22 genotypes of *D. sissoo*. According to them, RAPD markers were relatively more efficient than the ISSR assay. We used 30 arbitrary DNA primers to study variability in 29 clones of *D. sissoo*. Based on fingerprint profile, genetic relatedness among different genotypes of *D. sissoo* was determined.

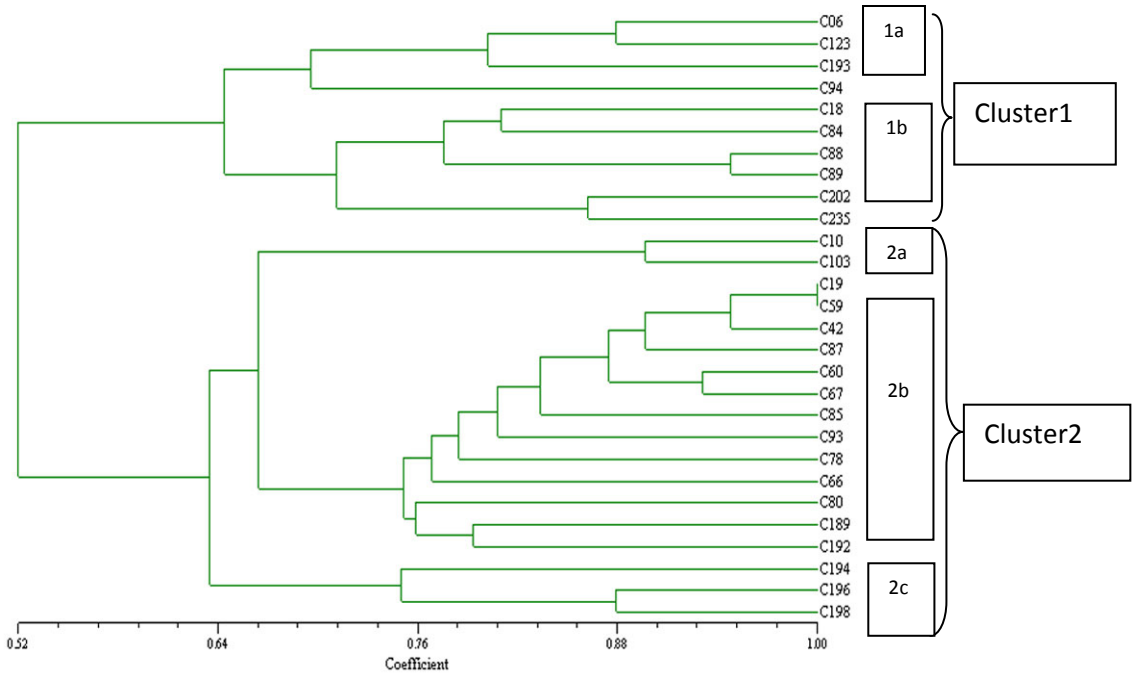

Fig. 2 UPGMA based dendrogram exhibiting genetic relationships among the clones of *Dalbergia Sissoo* using RAPD markers.

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